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Conditional-Suicide Containment System for Bacteria Which Mineralize Aromatics

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A model conditional-suicide system to control genetically engineered microorganisms able to degrade substituted benzoates is reported. The system is based on two elements. One element consists of a fusion between the promoter of the *Pseudomonas putida* TOL plasmid-encoded *meta*-cleavage pathway operon (P_m) and the *lacI* gene encoding Lac repressor plus *xylS*, coding for the positive regulator of P_m. The other element carries a fusion between the P_{tac} promoter and the *gef* gene, which encodes a killing function. In the presence of XylS effectors, LacI protein is synthesized, preventing the expression of the killing function. In the absence of effectors, expression of the P_{tac}::*gef* cassette is no longer prevented and a high rate of cell killing is observed. The substitution of XylS for XylSthr45, a mutant regulator with altered effector specificity and increased affinity for benzoates, allows the control of populations able to degrade a wider range of benzoates at micromolar substrate concentrations. Given the wide effector specificity of the key regulators, the wild-type and mutant XylS proteins, the system should allow the control of populations able to metabolize benzoate; methyl-, dimethyl-, chloro-, dichloro-, ethyl-, and methoxybenzoates; salicylate; and methyl- and chlorosalicylates. A small population of genetically engineered microorganisms became Gef resistant; however, the mechanism of such survival remains unknown.

A large number of xenobiotics have been introduced into the environment in recent years. Some of these compounds are readily degraded by microbes, while others are recalcitrant. Genetic engineering of catabolic pathways has proven to be an efficient technology by which to accelerate, in the laboratory, the evolution of biological pathways for the removal of recalcitrant compounds (see reference 16 for a review).

The TOL plasmid pWW0 of *Pseudomonas putida* specifies a pathway for the catabolism of toluene, xylenes, and related hydrocarbons via benzoates and toluates. The spectrum of alkyltoluenes and alkylbenzoates mineralized through TOL catabolic pathways was expanded by the genetic modification of existing barriers which prevented their metabolism (1, 17). TOL-encoded enzymes and regulators have also been employed to expand the range of chlorotoluenes and chlorobenzoates degraded by *Pseudomonas* sp. strain B13 (1, 18, 19).

The *meta*-pathway operon of plasmid pWW0 codes for enzymes allowing the transformation of benzoates and certain alkylbenzoates into Krebs cycle intermediates. Transcription of this operon requires the product of the *xylS* gene, which in the presence of 3-methylbenzoate or other benzoate effectors switches on transcription from P_m, the promoter of the pathway. The isolation of XylS mutants with altered effector specificities and/or higher affinities for effectors provided a useful mechanism for expanding the range of compounds susceptible to mineralization (14, 15, 17).

The exploitation of genetically engineered catabolic pathways to eliminate pollutants from the environment is a desirable objective. However, their utilization requires a containment system to prevent transfer of the manipulated genes to an indigenous population and possible damage to natural ecosystems through the establishment of foreign bacterial populations. One approach that has been proposed to control genetically engineered microorganisms (GEMs) is the use of the so-called suicide systems. Molin et al. (10) described a suicide system that allowed GEMs to survive only in the presence of tryptophan (conditional maintenance). The system was based on a gene encoding a cell-killing function (*hok*) fused to the *trp* promoter. Analogous systems for conditional death have been described (3) in which killing was achieved by inducing a *lac-hok* cassette with isopropyl-β-D-thiogalactopyranoside (IPTG). The *hok* gene from plasmid R1 (4) belongs to a family of genes (12) encoding small polypeptides (about 50 amino acids) which, when overexpressed, collapse the membrane potential and lead to cell death.

We report here the construction of a model suicide system that allows the bacterial population to survive only in the presence of effectors of the *meta*-cleavage pathway encoded by the TOL plasmid of *P. putida*. The system is based on the presence of key regulatory elements (P_m promoter and XylS transcriptional activator) from the *meta* pathway and the killing function provided by *gef*, a chromosomally encoded *Escherichia coli* gene highly homologous to *hok*. In this containment system, LacI protein expressed from a P_m::*lacI* fusion represses transcription from a P_{tac}::*gef* cassette in the presence of XylS effectors, whereas in the absence of XylS effectors, expression of the *gef* gene is no longer repressed, leading to cell killing. Substitution of XylS for mutant protein XylSthr45 expands the range of benzoate analogs, allowing survival of the "controlled" population.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *E. coli* strains used in this work were 71-18 (Δ*lac-pro* F′ *lacI*^{q} *lacZ*Δ*M15* *pro*$^+$ *supE*) (9) and CSH36 (*lacI*) (Cold Spring Harbor

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Laboratory). Strains were routinely grown in Luria-Bertani (LB) medium at 30°C, supplemented when required with ampicillin (200 μg/ml) and kanamycin (25 μg/ml). Unless otherwise indicated, XylS effectors were added to the culture medium to a concentration of 1 mM. IPTG was used at 100 μM.

Plasmids used in this work were as follows: pNM185, an IncP4 plasmid which encodes resistance to kanamycin and carries the *xylS* gene and the TOL *meta*-cleavage pathway operon promoter (P_m) (8); pERD2, similar to pNM185 but carrying the *xylS2* gene (encoding XylSthr45 protein) instead of *xylS* (15); and pHB101, a pKK223-3 derivative (Pharmacia) that confers ampicillin resistance and carries a P_{tac}::*gef* fusion, which was constructed by cloning between the *Eco*RI-*Hin*dIII sites of pKK223-3 a 0.5-kb *Hin*dIII-*Eco*RI fragment of pLKP118 bearing the *gef* gene (12) so that the *gef* gene was expressed from the P_{tac} promoter.

Construction of plasmids bearing a P_m::*lacI* fusion. Plasmids pCC100 and pCC102 were constructed so that a promoterless *lacI* gene was cloned downstream of P_m. First, a *lacI* gene from plasmid pTTQ19 (Amersham International) was amplified by using the polymerase chain reaction method. The oligonucleotides used for amplification (5′-GAGTGAATTCAGGGTGGTGA-3′ and 5′-GCCAGAGC TCCTAATGAGTGAGCTA-3′) were partly complementary to the ends of the *lacI* gene and were designed so that *EcoRI* and *SacI* sites would be generated near the ends of the amplified DNA fragment. The amplified *lacI* DNA was cleaved with *Eco*RI and *Sac*I enzymes and inserted into the *Eco*RI-*Sac*I site of pNM185 (8) so that a promoterless *lacI* gene would be in front of the TOL *meta*-cleavage pathway promoter P_m. The resulting plasmid was called pCC100.

The wild-type *xylS* gene in pCC100 was replaced by the *xylS2* gene to generate plasmid pCC102. For this purpose, the 3.6-kb *Eco*RI-*Hin*dIII fragment from plasmid pERD2 containing *xylS2* was cloned into plasmid pCC100 to replace the homologous *Eco*RI-*Hin*dIII fragment containing *xylS*.

Recombinant DNA techniques. General molecular biology techniques were performed, with minor modifications, essentially as described by Maniatis et al. (7). The conditions for the amplification of DNA were those described by Ausubel et al. (2). Transformation of *E. coli* was carried out by the $CaCl_2$ method (7).

Survival assay for bacteria bearing the containment system. CSH36(pCC100, pHB101) and CSH36(pCC102, pHB101) were grown overnight in LB medium with ampicillin, kanamycin, and 1 mM 3-methylbenzoate. Cells were harvested and washed in LB medium and spread on LB plates unsupplemented or supplemented with different benzoate analogs (1 mM). After 24 h of incubation at 30°C, the colonies were counted.

RESULTS

Rationale of the containment system. With the aim of connecting the presence of a chemical to be mineralized with the regulation of a killing gene in the bacteria, we have designed a containment system consisting of two elements (Fig. 1). One element carries the gene for the Lac repressor (*lacI*) under the control of P_m and the gene *xylS* (or *xylS2*) (coding for the regulator necessary to activate transcription from P_m in the presence of an effector such as 3-methylbenzoate). The second element comprises a fusion between the P_{tac} promoter, which is negatively regulated by the LacI protein, and the *gef* gene, which codes for a killing function. When both plasmids are present in a cell growing in the

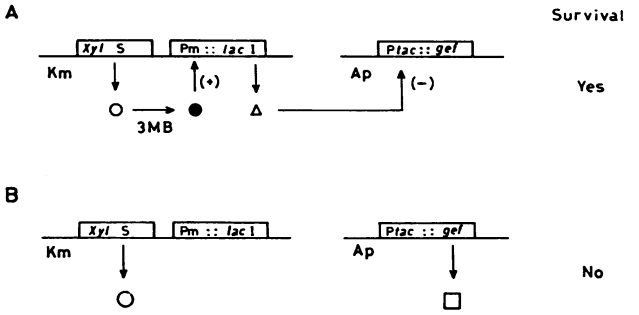

FIG. 1. Elements of conditional containment system based on the TOL plasmid regulator XylS, LacI, and a killing protein. The operation of the containment system is described in Results. (A) Conditions under which survival of the contained bacteria is predicted; (B) conditions under which it is predicted that bacteria bearing the containment system will die. 3MB, 3-Methylbenzoate. Symbols: ○ and ●, inactive and active forms of XylS protein, respectively; △ and □, LacI and Gef proteins, respectively.

presence of a XylS inducer, the LacI protein is synthesized, which prevents transcription from P_{tac} and synthesis of the Gef protein. In the absence of the *meta*-cleavage pathway effector, LacI synthesis will stop and transcription from P_{tac} will lead to the production of the Gef protein and cell death.

This defines a containment system for bacteria able to mineralize toxic organic compounds; the system is conditioned by the presence or absence of the pollutant in the environment and is designed to allow the survival of bacteria as long as the toxic compound is present in the environment, but not after it has been eliminated and not in locations outside the polluted area.

Expression of *lacI* under P_m promoter control. Plasmids pCC100 and pCC102 were constructed so that a promoterless *lacI* gene was cloned downstream of P_m. In the former construction, expression from P_m is mediated by the XylS protein once it is activated by benzoate effectors. In the latter construction, expression is mediated by XylSthr45, which differs from wild-type XylS by a single amino acid (Arg45→Thr) and is capable of recognizing benzoate effectors that are not recognized by XylS (15). Benzoates recognized by XylSthr45 but not by XylS include 2-hydroxy-, 4-ethyl-, and 2- and 4-methoxybenzoate. To test whether a functional LacI protein is expressed in these constructions, plasmids pCC100 and pCC102 were transformed into the *E. coli* $LacI^-$ strain CSH36. β-Galactosidase activity expressed from a chromosomally encoded *lacZ* gene was monitored on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) indicator plates. As expected, CSH36, CSH36(pCC100), and CSH36(pCC102) were all blue on X-Gal plates. In contrast, when 3-methylbenzoate was added to the culture medium, only colonies from CSH36(pCC100) and CSH36(pCC102) appeared colorless on X-Gal indicator plates, suggesting that the LacI protein was synthesized in response to the XylS effector. Furthermore, CSH36(pCC102) produced colorless colonies on X-Gal plates in the presence of specific XylSthr45 effectors such as 2-hydroxy- and 4-methoxybenzoate.

XylS-dependent induction of killing functions. To construct strains whose survival is dependent on the presence of a XylS or XylSthr45 effector such as 3-methylbenzoate, we used plasmid pHB101, a pKK223-3 derivative which confers ampicillin resistance to the host and carries the killing function provided by the *gef* gene under the control of the P_{tac} promoter. Transformation of pHB101 into CSH36 car-

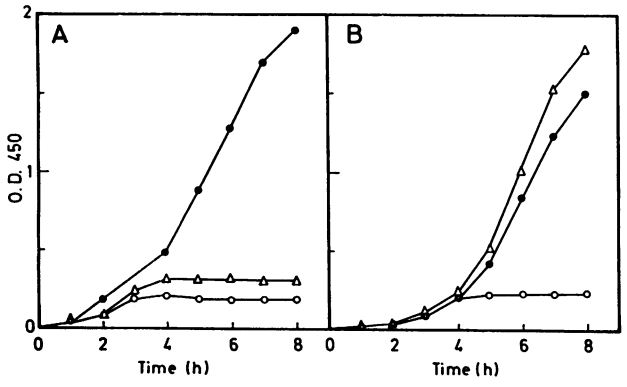

FIG. 2. Induction of killing in response to the absence of 3-methylbenzoate. (A) CSH36(pCC100, pHB101); (B) CSH36(pCC102, pHB101). Cultures were grown overnight in LB with ampicillin, kanamycin, and 3-methylbenzoate, washed, and diluted 1,000-fold in LB supplemented with ampicillin, kanamycin, and 1 mM (●) or 0.01 mM (△) 3-methylbenzoate or with ampicillin and kanamycin without 3-methylbenzoate (○). At the times indicated, turbidity was determined in a Perkin-Elmer spectrophotometer.

rying plasmids pCC100 or pCC102 gave a high frequency of Ap^r Km^r transformants on plates supplemented with 3-methylbenzoate. No transformants at all appeared on plates without a XylS effector or on plates supplemented with 3-methylbenzoate and IPTG (a LacI inactivator), indicating that the conditional killing system was functional and was coregulated in the anticipated manner.

Transfer of CSH36(pCC100, pHB101) and CSH36 (pCC102, pHB101) from a culture medium including 3-methylbenzoate to a medium without 3-methylbenzoate resulted in inhibition of cell growth (Fig. 2). Growth of CSH36(pCC102, pHB101) was possible in the presence of concentrations of 3-methylbenzoate as low as 1 to 10 μM, in contrast to CSH36(pCC100, pHB101), which requires concentrations of 3-methylbenzoate as high as 300 μM for growth. This difference probably reflects the fact that the protein encoded by the *xylS2* allele, the XylSthr45 regulator, has at least 50-fold-higher affinity for 3-methylbenzoate than the wild-type protein (14). It should, however, be noted that after overnight incubation of both strains in medium without effector, culture growth resumed (data not shown), suggesting that a small proportion of the cell population escaped control.

Induction of *gef* by different substituted benzoates. The ability of different substituted benzoates to prevent induction of the killing function was studied in bacteria carrying the conditional-suicide system. The results are shown in Table 1. CSH36(pCC100, pHB101) and CSH36(pCC102, pHB101) survived well in the presence of benzoates that are effectors for XylS and XylSthr45, respectively. Survival in the absence of effectors or in the presence of benzoates that are not XylS or XylSthr45 effectors was on the order of 10^{-5} to 10^{-6}.

4-Ethyl- and 2-methoxybenzoate are not substrates of the TOL plasmid catabolic pathway; nevertheless, they are both capable of activating the mutant protein XylSthr45 and are therefore able to prevent the killing of CSH36(pCC102, pHB101) but not of CSH36(pCC100, pHB101) (Table 1). Hence, survival of a bacterial population in which the containment system is incorporated and which is growing in the presence of XylS or XylSthr45 effector is mainly determined by the quality of the effector(s) and the type(s) of regulator present. The wider range of effectors for the XylSthr45 protein than for the wild-type protein adds to the system the possibility of survival in a broader range of substituted benzoates.

TABLE 1. Survival of CSH36(pCC100, pHB101) and CSH36(pCC102, pHB101) in the presence or absence of benzoates[a]

Benzoate	Survival frequency	
	XylS	XylSthr45
Benzoate	1	1
2-Methylbenzoate	1	1
3-Methylbenzoate	1	1
3-Chlorobenzoate	1	1
4-Ethylbenzoate	10^{-5}–10^{-6}	1
2-Methoxybenzoate	10^{-5}–10^{-6}	1
3-Hydroxybenzoate	10^{-5}–10^{-6}	10^{-5}–10^{-6}
None	10^{-5}–10^{-6}	10^{-5}–10^{-6}

[a] CSH36(pCC100, pHB101) and CSH36(pCC102, pHB101) were grown as described in Material and Methods. Serially diluted cultures were plated on LB plates containing ampicillin and kanamycin and supplemented with the indicated benzoate (1 mM). Colonies were counted after 24 h of incubation at 30°C. Data are the average of two to five independent experiments. Standard deviations were between 2 and 28% of the given values. Survival on different benzoates is relative to survival on 3-methylbenzoate.

Analysis of clones surviving *gef* induction. The appearance of "*gef*-resistant" colonies at a frequency of about 10^{-6} led us to investigate the phenotype as well as the genotype of such clones. Since all experiments described in this work were performed by directly selecting for ampicillin and kanamycin for the maintenance of the two plasmids forming the containment system, the loss of the plasmids can be ruled out. The first step, therefore, was to find out whether survival reflects a transient physiological resistance or a permanent genetic change. In the latter case, the mutation could be located (i) in the plasmid carrying the *xylS* gene and the P_m::*lacI* fusion, thus causing the constitutive synthesis of LacI; (ii) in plasmid pHB101, thus inactivating the *gef* gene; or (iii) in the bacterial chromosome, thus preventing either *gef* expression or the Gef killing function (immunity).

Strain CSH36(pCC100, pHB101) was plated on media without benzoate; 31 surviving colonies were transferred to plates unsupplemented or supplemented with 3-methylbenzoate. Five out of the 31 turned out to be "false" colonies, since they failed to grow even in media with the benzoate analog. Another five showed a "normal" induction pattern, suggesting transient resistance to *gef* expression. To rule out transient resistance in the remaining 21 colonies, they were transferred four consecutive times to media with 3-methylbenzoate and then tested for induction of killing. All these clones escaped killing, suggesting that they have "bona fide" mutations. In order to test whether the 21 killing-resistant clones carried a functional pHB101 plasmid, plasmid DNA was isolated from those clones and about 100 ng was transformed into *E. coli* 71-18, carrying a $lacI^q$ gene; selection was then performed for Ap^r transformants. In 16 out of 21 transformations, no Ap^r colonies were recovered, and the remaining 5 transformations produced between one and five Ap^r colonies. This is a strong indication that an active *gef* gene was still present in pHB101, since the amount of LacI protein synthesized by the F′ plasmid of strain 71-18 probably cannot allow full repression of the P_{tac}-*gef* cassette. Furthermore, all the Ap^r transformants recovered (a total of 10) showed IPTG-mediated induction of

gef. These results rule out a modification of the pHB101 plasmid as the cause of the *gef*-resistant phenotype in the colonies analyzed.

Strain CSH36 was transformed with the same amount of plasmid DNA from the 21 killing-resistant strains, and selection was performed on plates supplemented with kanamycin. Hundreds of Km^r colonies appeared in all 21 transformations, and all of them showed the same behavior as the control strain CSH36(pCC100) on X-Gal indicator plates without inducer, suggesting that a mutation affecting LacI activity was not involved in the phenotype of noninducible clones.

In short, the two plasmids forming the containment system seemed to be stable under selective conditions, and the host mechanisms to escape killing remain unknown.

DISCUSSION

In this study, we report the construction of a model containment system based on substrate availability. The system consists of two elements. The first contains a fusion of the P_{tac} promoter to the *gef* gene, which codes for a cell-killing function. Expression from P_{tac} is negatively controlled by the LacI protein. The second element is designed to control the first one and is composed of a fusion of the P_m promoter to a promoterless *lacI* gene plus the wild-type XylS protein or the mutant XylSthr45. XylS proteins stimulate transcription from P_m in the presence of benzoate effectors.

Bacteria bearing the P_{tac}::*gef* fusion are not viable unless they also contain the second element and are selected on media with an effector for the XylS or XylSthr45 regulators. Bacteria bearing both elements of the killing system grow in the presence of benzoate effectors at rates comparable to those of bacteria without the killing system. In contrast, transference of the bacteria to a medium without a benzoate effector results in the prevention of growth through cell killing. The XylSthr45 mutant protein exhibits increased affinity for benzoate effectors (at least 50-fold-higher affinity for methylbenzoates than is exhibited by the wild-type regulator) (14) and, in addition, can recognize as effectors substituted benzoates that do not activate the wild-type XylS protein. Among these are 2,4-dimethyl-, 2,4-dichloro-, 2-methoxy-, 3-methoxy-, 4-methoxy-, and 4-ethylbenzoate; salicylate; and methyl- and chlorosalicylates (15). The use in the second element of the killing system of a mutant regulator with altered effector specificity and increased affinity for effectors has two dramatic effects on the performance of the killing system. One effect is that the system functions at relatively low concentrations, i.e., in the micromolar range, and second effect is that the system can, in principle, be extended to control GEMs in which genes from the TOL plasmid have not been used, i.e., *Pseudomonas* sp. strain B13 (pPL300-1). This bacterium is able to degrade salicylates and chlorosalicylates and was constructed by recruiting the *Pseudomonas nahG* and *nahR* genes from the NAH7 plasmid (6). The facts that DNA sequences homologous to *gef* and *hok* genes have been found in very different gram-negative bacteria (12) and that R1 Hok protein is toxic for the gram-positive *Bacillus subtilis* (11) suggest that the containment system can, in principle, be used in a broad spectrum of bacteria.

Two important drawbacks of the system in its present state are the concentration of effector required to activate the XylS and XylSthr45 proteins and the appearance of clones resistant to killing, a circumstance previously observed (3, 10, 11). Although XylSthr45 exhibits at least 50-fold-higher affinity than XylS for 3-methylbenzoate, the concentrations required to activate the regulator (in the micromolar range) are still relatively high compared with environmental concentrations, and it would be desirable to isolate mutants with a higher affinity for effectors which can be isolated by modifying the original strategy used by Ramos and Timmis (13), thereby finding mutants with altered effector specificities. Regarding the second limitation, our data suggest that resistance to killing may be due to more than one cause, either to (i) transient resistance, which leads to a delay in the induction of killing, or to (ii) bona fide chromosomal mutations that, through some unknown mechanism, prevent the killing system from functioning. Therefore, the increase in the efficiency of killing requires further studies of the physiological role of the proteins involved in the induction of cell death. Moreover, more than a single killing system may be necessary to avoid the cells' escaping the killing functions.

To extend the use of the conditional-suicide system to other organisms, specific host killing functions may be required to achieve high rates of killing. In addition, work should be done toward introducing the killing functions on the chromosomes of GEMs or in plasmids bearing manipulated genes. This can be achieved by using transposaseless suicide transposons such as those described by Herrero et al. (5).

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REFERENCES

1. **Abril, M. A., C. Michan, K. N. Timmis, and J. L. Ramos.** 1989. Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. J. Bacteriol. **171**:6782–6790.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, New York.
3. **Bej, A. K., M. H. Perlin, and R. M. Atlas.** 1988. Model suicide vector for containment of genetically engineered microorganisms. Appl. Environ. Microbiol. **54**:2472–2477.
4. **Gerdes, K., P. B. Rasmussen, and S. Molin.** 1986. Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells. Proc. Natl. Acad. Sci. USA **83**:3116–3120.
5. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172**:6557–6567.
6. **Lehrbach, P. R., J. Zeyer, W. Reineke, H. J. Knackmuss, and K. N. Timmis.** 1984. Enzyme recruitment in vitro: use of cloned genes to extend the range of haloaromatics degraded by *Pseudomonas* sp. strain B13. J. Bacteriol. **158**:1025–1032.
7. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Mermod, N., J. L. Ramos, A. Bairoch, and K. N. Timmis.** 1987. The *xylS* gene positive regulator of TOL plasmid pWW0: identification, sequence analysis and overproduction leading to constitutive expression of *meta* cleavage operon. Mol. Gen. Genet. **207**:349–354.
9. **Messing, J., B. Gronengorn, B. Muller-Hill, and B. Hofschneider.** 1977. Filamentous coliphage M13 as a cloning vehicle: insertion of a *HindIII* fragment of the *lac* regulatory region in

M13 replicative form *in vitro*. Proc. Natl. Acad. Sci. USA **74**:3642–3646.

10. **Molin, S., P. Klemm, L. K. Poulsen, H. Biehl, K. Gerdes, and P. Anderson.** 1987. Conditional suicide system for containment of bacteria and plasmids. Bio/Technology **5**:1315–1318.
11. **Molin, S., P. Klemm, L. K. Poulsen, H. Biehl, K. Gerdes, and P. Anderson.** 1988. Biological containment of bacteria and plasmid to be released in the environment, p. 127–136. *In* W. Klingmuller (ed.), Risk assessment for deliberate releases. Springer-Verlag, Berlin.
12. **Poulsen, L. K., N. W. Larsen, S. Molin, and P. Anderson.** 1989. A family of genes encoding a cell-killing function may be conserved in all Gram-negative bacteria. Mol. Microbiol. **3**:1463–1472.
13. **Ramos, J. L., N. Mermod, and K. N. Timmis.** 1987. Regulatory circuits controlling transcription of TOL plasmid operon encoding *meta*-cleavage pathway for degradation of alkylbenzoates by *Pseudomonas*. Mol. Microbiol. **1**:293–300.
14. **Ramos, J. L., C. Michan, F. Rojo, D. Dwyer, and K. N. Timmis.** 1990. Signal-regulator interactions. Genetic analysis of the effector binding site of *xylS*, the benzoate-activated positive regulator of *Pseudomonas* TOL plasmid *meta*-cleavage pathway operon. J. Mol. Biol. **211**:373–382.
15. **Ramos, J. L., A. Stolz, W. Reineke, and K. N. Timmis.** 1986. Altered effector specificities in regulators of gene expression: TOL plasmid *xylS* mutants and their use to engineer expansion of the range of aromatics degraded by bacteria. Proc. Natl. Acad. Sci. USA **83**:8467–8471.
16. **Ramos, J. L., and K. N. Timmis.** 1987. Experimental evolution of catabolic pathways of bacteria. Microbiol. Sci. **4**:228–237.
17. **Ramos, J. L., A. Wasserfallen, K. Rose, and K. N. Timmis.** 1987. Redesigning metabolic routes: manipulation of TOL plasmid pathway for catabolism of alkylbenzoates. Science **235**:593–596.
18. **Reineke, W., and H. J. Knackmuss.** 1979. Construction of haloaromatics utilizing bacteria. Nature (London) **227**:385–386.
19. **Rojo, F., D. Piepper, K. H. Engesser, H. J. Knackmus, and K. N. Timmis.** 1987. Assemblage of *ortho*-cleavage route for simultaneous degradation of chloro- and methylaromatics. Science **238**:1395–1398.